REMARKS

Claims 18, and 20-34 are in this application. Claim 19 has been cancelled. Claims 31-34 are withdrawn. Claims 18, and 20-29 are amended as suggested by the Examiner.

Page 30 of the specification has been amended to delete the hyperlink. It is applicants' position that replacement of the hyperlink to the Cleveland Clinic's website with the phrase "See the website of the Cleveland Clinic" is not new matter because the same information was conveyed by the original disclosure of the website address.

The English translation of the priority application P200400163 was filed on June 17, 2009 as shown in PAIR. The certified copy of the priority application was filed during the international phase of PCT/IB05/00187 and this is shown by the attached page from the WIPO website.

According to page 3 of the Office Action, the Examiner considers that claims 18-21, 27, 29 are anticipated by Spano et al. This is respectfully traversed.

Anticipation requires that each and every element of the claimed invention be disclosed in a single prior art reference. *In re Paulsen*, 30 F.3d 1475, 31 USPQ 1671 (Fed. Cir. 1994). For anticipation, there must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. *Scripps Clinic & Res. Found. v. Genentech, Inc.*, 927 F.2d 1565, 18 USPQ2d 1001 (Fed. Cir. 1991).

As will be explained below, Spano et al. does not disclose each element of the claims and therefore, Spano cannot and does not anticipate claims 18-21 and 27-29.

Claim 18 of this application claims a method comprising:

a) treating a sample containing the sperm, with a solution of DNA denaturing solution,

b) a single treatment step of treating the sample in the solution obtained in step a) with a lysis solution to extract nuclear proteins of the sperm cells, wherein the lysis solution does not contain protein denaturing detergents, and

c) evaluating the integrity of the chromatin/DNA of the sperm cells based on measurement of halo size of the sperm cells.

The method comprises three sequential steps:

Step 1) Denaturing step, using a DNA denaturing solution

Step 2) Lysis step, using a single lysis solution, and

Step 3) Evaluation step.

The treatment of the sample comprises two sequential steps, a denaturing step and a lysis step, each of them using a different solution.

Spano et al. discloses a method to study the sperm chromatin quality, both before and after swim-up and after cryopreservation. For this end, Spano et al use the sperm chromatin structure assay (SCSA), already described by Evenson and Jost (1994) (see page 30, first line under "flow cytometric analysis" subtitle). This reference, 'Evenson and Jost', is also cited on page 3, line 3 of this application.

The SCSA is a flow cytometric (FCM) technique which exploits the methachromatic properties of Acridine Orange (AO) to monitor the susceptibility of sperm chromatin DNA to an in situ acid partial denaturation. This method consists of 1) treating the sample with a denaturing detergent solution (0.17% Triton x 100, 0.15 M NaCl, 0.08 N HCl, pH 1,4) (see page 30, col. 2, line 9-12) and 2) staining the sample with AO.

Therefore, prior to the staining step, only one treatment step (a denaturing step with a denaturing solution) is carried out. There is not a subsequent lysis step, using a lysis solution, as in the present invention.

Applicants respectfully content that the Examiner has misinterpreted the content of Spano et al. On page 4 of the action, the Examiner considers that the use of a denaturing solution is disclosed in page 30, col.2, lines 8-9 of the paragraph under the subtitle "flow cytometric analysis...". The Examiner considers that the use of a lysis solution is supported in page 30, col.2 lines 9-12 of the paragraph under the sub title "flow cytometric..). The complete paragraph (lines 8-12) recites as follows:

> "After thawing on crushed ice, sperm cells were subjected to partial denaturation of DNA in situ and then stained with AO.
> 0.2 ml aliquots ([of the sample]) were admixed with 0.4 ml of a low pH detergent solution (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4). After 30 s, the cells were stained by adding 1.2 ml of a solution..."

As it can be clearly deduced from this paragraph, the detergent solution is used in the partial denaturation step. So that, only <u>one treatment step</u> (denaturing), using only <u>one solution</u> (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4), is carried out before staining the sample. There is not a lysis step.

This can also be deduced from the background of the present application, wherein it is disclosed that, in 'Evenson et al', "the sperm in suspension are added to an acid denaturing solution...They are then stained with Acridine Orange (page 3, lines 7-8). Evenson does not disclose a lysis step.

Further, in the communication (page 4), the Examiner cites that an evaluation step based on the measurement of the halo size is disclosed in page 30 and page 31 of Spano et al. However, this document was reviewed and there is no disclosure of halo size measurements.

In the 'Spano et al' method, the treated sample is stained with AO and evaluated by flow cytometry. This AO staining emits a green fluorescence when it binds with double stranded DNA while, in the sperm with denatured DNA, in single strand, this fluorochrome emits a red

fluorescence. Therefore, the sperm with denatured DNA are quantified using flow cytometry, to discriminate between both types of fluorescence (page 30 and 31, under subtitle "Flow cytometric analysis...").

The method of the present invention methacromatic staining, as AO is not required nor is it required to used flow cytometry to discriminate between different types of fluorescence.

In the present invention, the evaluation step is a direct visual analysis, based on the measurement of the halo sizes. The essential features of the method of the invention, the use of a mild two sequential steps and a mild lysis solution, allow a better preservation of the morphology of the head, or core, obtaining dispersion halos. Further, the tail is maintained, allowing differentiating sperm cells from other kind of cells. The evaluation can be carried out routinely, by conventional microscopy.

In the background section of the present application, those differences between the method of the present invention and the method of Spano et al. (Evenson et al.) are detailed (page 3, lines 1-27). Further, the advantages derived from the method of the present invention as compared with Evenson et al technique are also disclosed.

Therefore, to summarize, , there are important differences between both methods:

1. The present invention comprises two sequential treatment steps of the sample:

- a denaturing step (using a denaturing solution)
- a lysis step (using a lysis solution).

Spano et al comprises only one treatment step (a denaturing step using a denaturing solution).

2. The present invention comprises an evaluation step based on the measurement of the halo size.

Spano et al. comprises an evaluation step based on the use of a methacromatic staining (AO), and flow cytometry to discriminate between different types of fluorescence.

Therefore, since each element of the claim is not disclosed in Spano, it is respectfully requested that the rejection be withdrawn.

The Examiner states claims 18-21, 24-27 and 29 are anticipated by Januskauskas et al. This is respectfully traversed.

This document defines a method to assess the sperm chromatin quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from bulls.

The technique used in the method disclosed in this document is the same as the method described in the Spano et al. (see page 951, reference 34 of Januskaukas et al.). Like Spano there is no disclosure of a lysis step or measuring of halo size in Januskaukas. The disclosure of Triton X-100 is in connection with the denaturing step as semen samples are mixed with the low pH detergent solution that contains both Triton and HCl.

Again, since each element of the claims is not disclosed in the reference, the reference cannot and does not anticipate claims 18-21, 24-27 and 29 and it is respectfully requested that this rejection be withdrawn.

The Examiner considers that claims 18, 20-23, and 27-30 are anticipated by Connell et al. This is respectfully traversed.

Firstly, since the certified copy of the priority application and a certified English translation of the application have been filed, Connell et al. is not prior art and not properly cited.

Secondly, Connell et al. describes an assay to determine the mitochondrial DNA and nuclear DNA fragmentation of sperm populations separated by using discontinuous density gradient. As it is cited in page 756 (2nd column, first paragraph), the methodology used to

determine the DNA integrity was carried out through a procedure previously described in the document "Donnelly et al". Thus, as in that document, the integrity of the DNA was determined through single cell alkaline gel electrophoresis (COMET) assay. In this assay, cells were lysed with a cold lysis solution (NaCl, Na_2EDTA, Tris y Tritón X-100) for 1 h at 4°C. Later, cells were incubated for 30 minutes at 4°C in DTT, following by 90 minutes incubation at 20°C with lithium diiodosalicyclate (LIS) in order to decondense the DNA.

Donnelly et al. was cited by the Examiner in the Office Action of September 26, 2008 as both anticipating and making obvious the claimed invention. On pages 5-8 of applicants' response to the Office Action of September 26, 2008, it was clearly explained why this reference did not anticipate nor make obvious the claims. The Examiner states in paragraph 2 of this action "All objections and rejections that are not reiterated herein are withdrawn in view of the amendment and persuasive arguments." Since the disclosure that the Examiner is relying on in citing Connell et al. is the same as the disclosure of Donnelly et al. that was distinguished in the response to the previous Office Action, it is respectfully requested that this rejection be withdrawn.

It is submitted that the application is in condition for allowance and favorable consideration is respectfully requested.

Respectfully submitted,

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